Division of Dockets Management (HFA-305),
U.S. Food and Drug Administration,
5630 Fishers Lane, Rm. 1061,
Rockville, MD 20852.
USA

Lyngby 21st July 2005

Re: risk assessment of *Listeria monocytogenes* in smoked finfish

(Docket No. 2005N-0065, Risk Assessment of the Public Health Impact from Foodborne *Listeria monocytogenes* in Smoked Finfish; and Evaluation of Food Code Provisions that Address Preventive Controls for *Listeria monocytogenes* in Retail and Foodservice Establishments).

Comments and data provided by the Danish Institute for Fisheries Research, Department of Seafood Research. Submitted by **L. Gram** (gram@difres.dk). Unpublished data have been provided by **B.F. Vogel** (bfv@difres.dk)

Introductory remarks:

The product smoked fish covers a heterogeneous group of products. Hence for each of the eight areas where information is requested, one must consider:

* is the fish hot or cold-smoked?,
* how is the fish cold-smoked (traditional (slow) ovens or rapid ovens or liquid smoke)
* to what degree is the fish smoked (level of phenols)
* how is the fish salted (dry salting or brine or injection)
* what are the NaCl-levels (vary from 2 to 7% NaCl in water phase),
* what are lactate levels,
* what are the levels of lactic acid bacteria
* if challenge trials were conducted, how were *Lm* pre-cultured? how was it inoculated into/onto the food?

The inactivation of *Lm* during processing and the potential growth of *Lm* in a smoked product depends on all of the above factors. Most studies have, logically, been carried out as challenge trials where *Lm* has been inoculated on the product. As demonstrated by Dalgaard and Jørgensen (1998) the growth in naturally contaminated products may be significantly slower.

The model used for *Lm* in smoked fish in the WHO/FAO risk assessment incorporates some of these variables (see also Ross et al. 2000, Gimenez and Dalgaard 2004).

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Lone Gram

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1. *Lm* levels in raw fish, smoked fish and finished product

Raw fish (fish directly out of water):

When sampling from raw fish (marine or freshwater) taken directly out of the water – before any handling, we have rarely detected any *L. monocytogenes*.

In Wulff et al. (2005) we checked nine samples (all pooled from five salmon/sea-water trout) and found none positive for *Lm*. fish were swabbed on skin and on gills. In Hansen (2005) [M.Sc. thesis – not yet defended - will be available later this year] we sampled 12 fish from seawater and found none positive for Lm.

In a recent M.Sc. Thesis (Hansen 2005), we sampled 24 rainbow trout from freshwater fish farms. We detected *Lm* on 2 of 24 fish samples.

In Ben Embarek et al. (1997), 10 fish were sampled – and gills, skin and guts tested separately for presence of *Lm* (or *Listeria* spp.) none were positive for *Listeria* spp. (or *Lm*)

Due to the extremely low prevalence, we have never estimated levels. It is reasonable to assume that they are very low (< 1 cfu/25 g), when present.

Smoked fish and processing facilitites

Prevalence in DK smoked salmon is currently (2005) very similar to data reported from the US (Gombas et al. 2003) where 2-4% of sampled product is positive for *Lm*. Wulff et al. (2005) sampled 51 samples of smoked salmon and found 4% positive for *Lm*. This prevalence varies between smoke houses as some run positive samples less than 0.5% whereas others are higher at approx. 10%. We have sampled 23 non-salmon smoked products and found 48% positive (Tables 1 and 2).

Lm levels in smoked salmon were investigated by Jørgensen and Huss (1998) who found that 28% contained less than 10 *Lm*/ g. This was in a study in which 34% were positive right after packaging. As is clear, the prevalence in DK (and other) smoked fish products has gone down dramatically. We have not recently investigated levels but it is reasonable to assume that is it lower than reported in 1998.

Jørgensen and Huss (1998) also investigated levels of Lm after 3-8 weeks of storage of commercial product at 5°C. 43% were positive; 17% contained less than 10 *Lm*/g, 11% between 10 and 100 *Lm*/g and 4 samples (6%) between 100 and 10,000 *Lm*/g. No sample had exceeded 10^4 Lm/g.

Clearly, the US data demonstrate that sporadically high levels can be found. It is a very great pity that the Gombas et al (2003) study did not publish the chemical characteristics of the samples with high levels. Were these samples with low NaCl-levels?

Table 1. Distribution on sample type and on zones of total numbers of samples collected, and *Listeria monocytogenes* positive samples from Smokehouse 1 - 4. Summarized numbers are written in bold. Note that sampling sites were not random but selected as being ones likely to harbour *Lm*. Samples taken after sanitization were sites where *Lm* was found before cleaning and disinfection. Wulff et al. (in preparation). To be handled confidentially.

Smoke-house	Sampling site	No. of samples		No. of Lm positive		% of Lm positive	
		Produc-tion	Sanitiza-tion	Produc-tion	Sanitiza-tion	Produc-tion	Sanitiza-tion
1	**Unprocessed fish**	**5**		**3**		**60**	
	Processing area [1)]	**105**	**44**	**17**	**4**	**16**	**9**
	Zone 1[1)]	28	11	4	1	14	9
	Zone 2	58	27	10	2	17	7
	Zone 3	19	6	3	1	16	17
	Products	**24**		**4**		**17**	
	Smoked salmon	12		0		0	
	Other products	12		4		33	
2	**Unprocessed fish**	**1**		**1**		**100**	
	Processing area [1)]	**78**	**79**	**25**	**13**	**32**	**16**
	Zone 1	22	9	6	1	27	11
	Zone 2	48	60	14	10	29	17
	Zone 3	8	10	5	2	63	20
	Products	**20**		**4**		**20**	
	Smoked salmon	16		1		6	
	Other products	4		3		75	
3	**Unprocessed fish**	**1**		**0**		**0**	
	Processing area [1)]	**100**	**47**	**32**	**13**	**32**	**28**
	Zone 1	27	3	2	1	7	33
	Zone 2	52	29	17	10	33	34
	Zone 3	21	15	13	2	62	13
	Products	**10**		**1**		**10**	
	Smoked salmon	8		1		13	
	Other products	2		0		0	
4	**Unprocessed fish**	**5**		**1**		**20**	
	Processing area [1)]	**96**	**51**	**19**	**8**	**20**	**16**
	Zone 1	19	9	2	0	11	0
	Zone 2	57	28	10	4	18	14
	Zone 3	20	14	7	4	35	29
	Products	**20**		**4**		**20**	
	Smoked salmon	15		0		0	
	Other products	5		4		80	
Total	**Unprocessed fish**	**12**		**5**		**42**	
	Processing area [1)]	**379**	**221**	**93**	**38**	**25**	**17**
	Zone 1	96	32	14	3	15	9
	Zone 2	215	144	51	26	24	18
	Zone 3	68	45	28	9	41	20
	Products	**74**		**13**		**18**	
	Smoked salmon	51		2		4	
	Other products	23		11		48	

[1)] Zone 1 = product contact surfaces, zone 2 = surfaces close to product, zone 3 = surfaces away from product.

Table 2. Distribution on sample type and on zones of total numbers of samples collected, and *Listeria monocytogenes* positive samples from Slaughterhouse A – D. Summarized numbers are written in bold. Note that sampling sites were not random but selected as being ones likely to harbour *Lm*. Samples taken after sanitization were sites where *Lm* was found before cleaning and disinfection. Wulff et al. (in preparation). To he handled confidentially

Slaughter-house	Sampling site	No. of samples		No. of Lm positive		% of Lm positive	
		Produc-tion	Sanitiza-tion	Produc-tion	Sanitiza-tion	Produc-tion	Sanitiza-tion
A	**Unprocessed fish**	**2**		**0**		**0**	
	Processing area	**52**	**66**	**26**	**18**	**50**	**27**
	Zone 1[1)]	25	28	11	5	44	18
	Zone 2	17	27	7	7	41	26
	Zone 3	10	11	8	6	80	55
	Fish in process	**2**		**2**		**100**	
B	**Unprocessed fish**	**3**		**0**		**0**	
	Processing area	**20**	**20**	**5**	**2**	**25**	**10**
	Zone 1	10	9	3	1	30	11
	Zone 2	4	5	1	1	25	20
	Zone 3	6	6	1	0	17	0
	Fish in process	**3**		**0**		**0**	
C	**Unprocessed fish**	**1**		**0**		**0**	
	Processing area	**24**	**21**	**16**	**12**	**67**	**57**
	Zone 1	5	9	5	6	100	67
	Zone 2	9	8	6	3	67	38
	Zone 3	10	4	5	3	50	75
	Fish in process	**5**		**1**		**20**	
D	**Unprocessed fish**	**3**		**0**		**0**	
	Processing area	**21**	**19**	**5**	**1**	**24**	**5**
	Zone 1	10	6	2	0	20	0
	Zone 2	7	11	2	0	29	0
	Zone 3	4	2	1	1	25	50
	Fish in process	**5**		**1**		**20**	
Total	**Unprocessed fish**	**9**		**0**		**0**	
	Processing area	**117**	**126**	**52**	**33**	**44**	**26**
	Zone 1	50	52	21	12	42	23
	Zone 2	37	51	16	11	43	22
	Zone 3	30	23	15	10	50	43
	Fish in process	**15**		**4**		**27**	

[1)] Zone 1 = product contact surfaces, zone 2 = surfaces close to product, zone 3 = surfaces away from product.

2. Effect of mitigation procedures on reduction of *Lm* in raw fish and finished product

We have (unpublished data) inoculated raw salmon blocks with *Lm* reaching approx. 1000 cfu/g and thereafter washed/dipped the blocks in a range of solutions: water, citric acid, lactate etc. using from 0 to 5%. No reduction is seen at concentrations of the compounds that do not affect the appearance of the product. Concentrations that allow a reduction of *Lm*, however, will leave the fish with a cooked appearance (denatured protein) not suitable for further processing.

Experiments with e.g. high pressure treatment after cold-smoking and packaging (Lakshmanan and Dalgaard 2004) have not been successful. Treatments that reduced *Lm* counts results in change of colour and texture.

3. Transfer of *Lm* from contaminated surfaces to product

We have not data on levels of *Lm* in products and the potential level in contaminated processing environments. Isolating *Lm* from the processing environment and from the finished product has been done in several Finnish, Danish and US studies. All indicate that the major (immediate) source of contamination is the environment.

4. Transfer of *Lm* from slicer to product

We have no quantitative data. Sub-typing of *Lm* from process-environment and product has indicated that the slicer can be a very important source of contamination (Vogel et al. 2001b).

In one plant (unpublished data) did we find *Lm* in the inside of the needles used for brine injection. The plant used saturated (approx. 25% NaCl) brine – and the sub-type of Lm contaminating the product was found inside the needles. Subsequent procedures involved steaming the needles at least once a week. This removed the source of contamination.

5. Impact of adding inhibitors to the product

Some bacteriocins may inhibit growth of *Lm* in smoked fish. Our data (Nilsson et al. 1997) demonstrate that addition of nisin to a salmon just inoculated with *Lm* caused an immediate reduction of the organism but growth resumed at normal growth rate following a lag phase. later data (unpublished) has demonstrated that nisin is unlikely to remain active as it binds (reversibly) to the fish product and a simple water extract contains on bacteriocin activity. In contrast, bacteriocins from carnobacteria remain active.

We have in several studies demonstrated that the addition of live (non-pathogenic, non-spoiling) lactic acid bacteria can slow or eliminate growth of Lm in smoked product (Nilsson et al. 1999, 2004, Alves et al. 2005).

Lactate and diacetate at the levels used in e.g. frankfurters will inhibit growth of fish-derived *Lm* in broth systems (unpublished data). Cold-smoked salmon (prepared with NaCl only) was minced and mixed with lactate and diacetate (or lactate and acetate) and inoculated with two different isolates of *Lm* from a smoke house (Figure F106). Samples were vacuum-packed and stored at 10°C. Samples were taken regularly for enumeration of *Lm*. It is mandatory to store such ready-to-eat products at 5°C in Denmark, however trials were carried out at 10°C to simulate abuse temperatures.

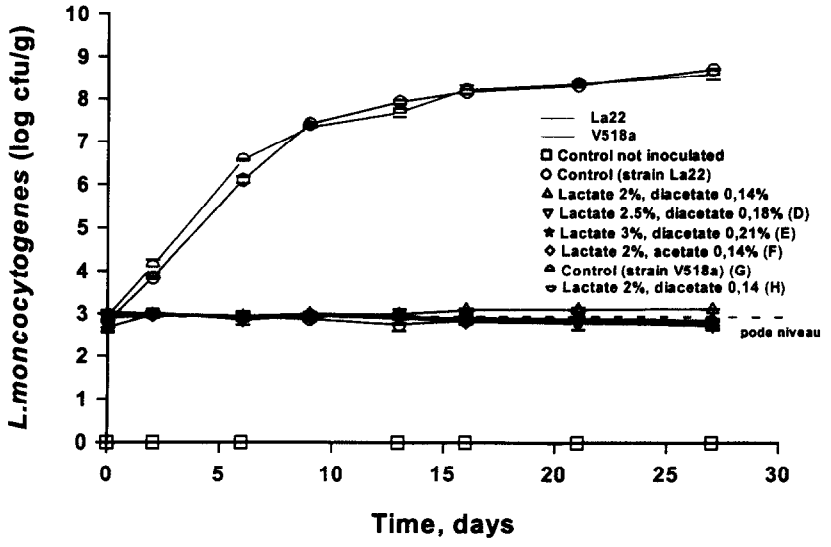


Figure F106. Growth of *Listeria monocytogenes* (strains La22 or V518a) in minced cold-smoked salmon mixed with lactate, diacetate or acetate. Samples were vacuum-packed and stored at 10°C

As is evident (Figure F106), lactate and diacetate prevented growth of *Lm*. The two strains both originate from fish smoke houses and were inhibited to the same degree.

Subsequently, experiments have been carried out to incorporate the preservatives in the cold-smoked fish. The brine (for brine injection) has been supplemented with lactate/diatetate (Purasal). Since lactate/diacetate decrease the solubility of NaCl, the NaCl-concentration in the mixed brine is lower than in the "normal" brine. Therefore, also fish brined with less salt (the control for the lactate/diacetate addition) has been included. The table below characterizes the 6 samples of fish produced. PA4 is Purasal product (lactate + acetate) and PD4 is Purasal product (lactate + diacetate)

Table 3: Characterization of cold-smoked salmon brine injected with NaCl or NaCl + purasal

Sample no	Description	NaCl %(WPS)	pH	Lactate %w/w	Acetate/diacetate % w/w
1	Control; normal	4.9	6.2	0.82	0.00
2	+ 2% PA4	3.7	6.2	1.99	0.12
3	+2% PD4	3.4	6.1	2.06	0.12
4	+1.5% PD4	4.3	6.2	1.90	0.09
5	Control, less NaCl	3.8	6.2	0.8	0.00
6	+2.7% PA4	2.7	6.2	2.27	nd

Minced smoked fish from these fish were inoculated with *Lm* mixed into the minced meat, vacuum-packed and stored at 10°C. Samples were taken at regular intervals for enumeration of *Lm*. The combination of 2% lactate and 0.12% diacetate completely prevented growth of *Lm* at 10°C. In contrast, combining with acetate at the same level did not completely prevent growth but did result in a slower growth (Figure F110). Interestingly, the growth in fish with 4.9% NaCl was equal to growth in the less salted fish (3.8% NaCl).

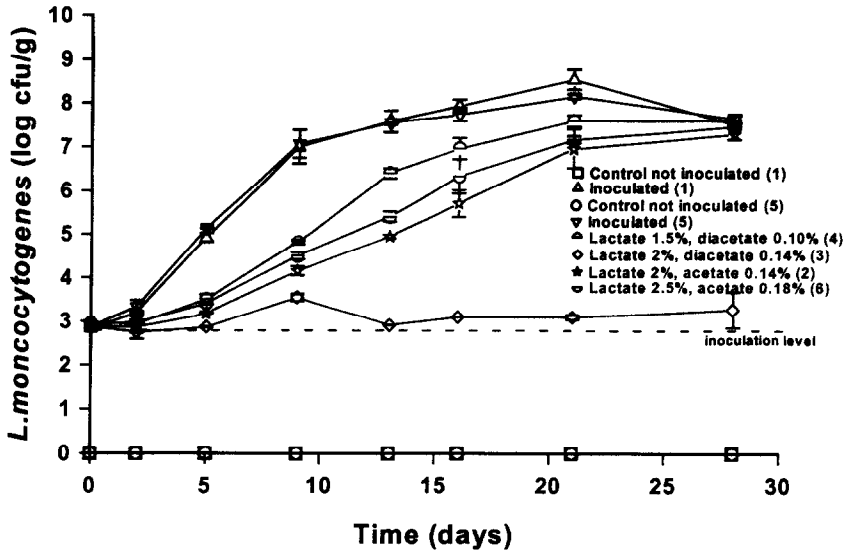
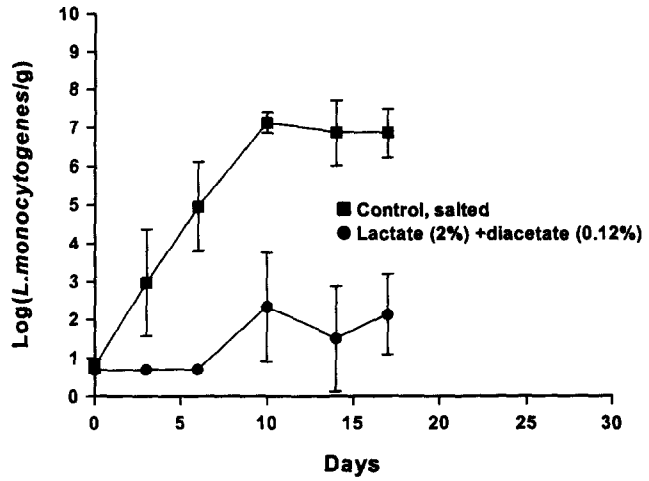


Figure F110. Growth of *Listeria monocytogenes* (strain La22) in minced cold-smoked salmon brine injected with NaCl, lactate and diacetate or acetate (Purasal product). Samples were vacuum-packed and stored at 10°C.

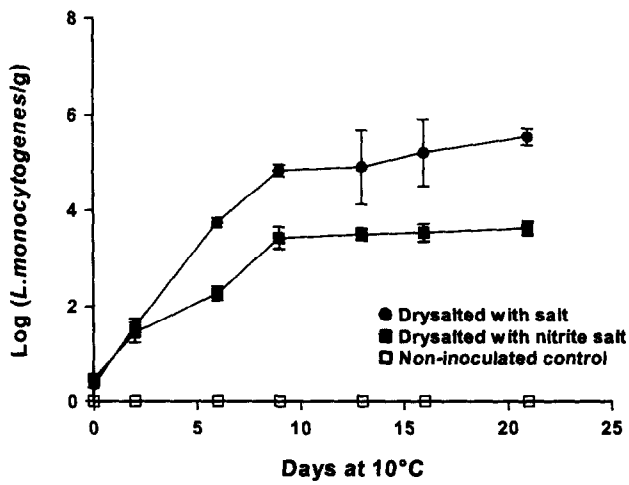
An on-going trial investigates the effect of a) a lower inoculum level and b) inoculating directly on slices (rather than homogenized product). Figure F119 demonstrates that lactate and diacetate continue to control growth of *Lm*.

Figure F119. Growth of *Listeria monocytogenes* (strain La22) in slices of cold-smoked salmon drysalted with NaCl or NaCl+lactate+diacetate. Samples were vacuum-packed and stored at 10°C.



Nitrite is used by some smoke-houses in the salting process for cold-smoked fish. Nitrite is not allowed as additive for products on the Danish market but is allowed in e.g. the US. In on-going experiments we are evaluating the possible growth inhibitory effect of the nitrite-addition. In an on-going trial, in which the nitrite-salted fish is minced and inoculated with *Lm*, we demonstrate that *Lm* grows (as expected) to almost 10^6 cfu/g in 21 days (from an inoculum of 5 cfu/g). In the nitrite-salted fish, *Lm*-growth is somewhat slower and levels off at 5 x 10^3 cfu/g (Figure F118). We have not yet measured concentrations of nitrite and NaCl.

Figure F118. Growth of *Listeria monocytogenes* (strain La22) in minced cold-smoked salmon drysalted with NaCl or NaCl+nitrite. Samples were vacuum-packed and stored at 10°C.



6. Impact of frozen versus refrigerated storage on levels of *Lm*

Frozen storage will eliminate growth of *Lm*. Preliminary data indicate that levels of *Lm* may decline slightly during frozen storage. The shell-freezing procedure used by some manufacturers immediately after cold-smoking to ease subsequent slicing has (in combination with the salting and smoking) a bacteria-reducing effect (Table 4). We have no data specifically on *Lm*.

Table 4. Aerobic colony count on Long and Hammer's medium from raw salmon, salted+smoked+shellfrozen salmon and sliced smoked salmon. The experiments have been conducted at two processing plants with raw materials stored for long or short periods.

Salting	% NaCl (WPS)	Log (cfu/g)			
		raw	salted/smoked/ shell-frozen	sliced	frozen storage
Injection	2.7	3.5	2.9	2.6	
Injection	3.7	6.3	4.6	3.8	3.7
Dry-salting	4.3	4.8	3.8	2.2	
Dry-salting	5.3	4.7	2.9	2.9	

7. Impact of time/temperature on levels of *Lm* for commercial and home storage conditions

No data. Except for the obvious that 10°C results in significantly faster growth than 5°C.

8. Effect of training regarding sanitation / hygienic practices on reducing the level of *Lm* in smoked fish

We have sampled from the processing environment of many fish smoke houses. We do look for sites where we assume *Lm* could hide. This results in 15-30% positive samples during processing which is similar to levels found in other studies. However, one processor runs at 4% and we have never seen a positive product sample from this smoke house. One major difference between this processor and all others is a tremendous staff stability (several have been there for 20 years), that the staff also takes care of cleaning - and that they are very much aware that *Lm* is a problem. This indicates that staff behaviour and their hygienic routines can play a role in Lm control. Also, however, this particular smoke houses has a relatively small production unit and their processes (raw material control, dry salting procedure) are carefully controlled.

It should be noted, that we have only paid one visit (sampling) to this particular smoke-house, whereas the other smoke-houses (e.g. table 1) have been visited and sampled on several occassions.

Relevant literature from DIFRES containing data on prevalence and growth of Lm in smoked product and other lightly preserved fish products:

Alves, V.F., E.C.P. de Martinis, M.T. Destro, B. Fonnesbech Vogel and L. Gram 2005. Antilisterial activity of a *Carnobacterium piscicola* isolated from Brazillian smoked fish [Surubim (*Platystoma* sp.)] and its activity against a persistent *Listeria monocytogenes* isolated from surubim. *J. Food Prot.* (accepted)

Bagge-Ravn, D., K. Gardshodn, L. Gram and B. Fonnesbech Vogel 2003. Comparison of fog and foam sanitizing procedures in a salmon smokehouse with respect to the general hygienic level and survival of *Listeria monocytogenes*. *J. Food Prot.* **66**, 592-598.

Ben Embarek, P.K. 1994. Presence, detection and growth of *Listeria monocytogenes* in seafood. A review. *Int. J. Food Microbiol.* **23** 17-34

Ben Embarek, P.K., L.Truelstrup Hansen, Ø. Enger and H.H. Huss 1997. Occurrence of *Listeria* spp. in farmed salmon and during subsequent slaughter: Comparison of the Listertest Lift and the USDA method. *Food Microbiol.* **14** 39-46

Bruhn, J.B., B. Fonnesbech Vogel and L. Gram 2005. Bias in the enrichment procedure for *Listeria monocytogenes:* lineage 2 strains outcompete lineage 1 strains in University of Vermont Broth. *Appl. Environ. Microbiol.* **71**, 961-967.

Dalgaard, P. and L.V. Jørgensen 1998. Predicted and observed growth of *Listeria monocytogenes* in inoculated seafood and in naturally contaminated cold smoked salmon. *Int. J. Food Microbiol.* **40** 105-116

Giménez, B. and P. Dalgaard 2004. Modelling and predicting the simultaneous growth of *Listeria monocytogenes* and spoilage microorganisms in cold-smoked salmon. *J. Appl. Microbiol.* **96**, 96-109.

Gram, L. 2001. Potential hazard in cold-smoked fish: *Listeria monocytogenes*. Special supplement to *J. Food Sci.* **66:**S1072-S1081

Himelbloom, B., L. Nilsson and L. Gram 2001. Factors affecting production of a bacteriocin produced by an antilisterial *Carnobacterium piscicola*. *J. Appl. Microbiol.* **91**, 506-512

Jeppesen, V.F. and H.H. Huss 1993. Antagonistic activity of two strains af lactic acid bacteria against *Listeria monocytogenes* and *Yersinia enterocolitica* in a model fish product at 5EC. *Int. J. Food Microbiol.* **19** 179-186

Jeppesen, V.F. and H.H.Huss 1993. Characteristics and antagonistic activity of lactic acid bacteria isolated from chilled fish products. *Int. J. Food Microbiol.* **18** 305-320

Jørgensen, L.V. and H.H. Huss 1998 Prevalence and growth of *Listeria monocytogenes* in Danish Seafood. *Int. J. Food Microbiol.* . **42**, 127-131

Jørgensen, L.V., P. Dalgaard, and H.H. Huss 2000. Multiple compound quality index for cold-smoked salmon (*Salmo salar*) developed by multivariate regression of biogenic amines and pH. *J. Agri. Food Chem.* **48** 2448-2453.

Lakshmanan, R. and P. Dalgaard 2004. Effect of high-pressure processing on *Listeria monocytogenes*, spoilage microflora and multiple compound quality indices in chilled cold-smoked salmon. *J. Appl. Microbiol.* **96**, 398-408.

Martinez, I., L.-M. Rørvik, M. Seppola, V. Brox, J. Lassen, L. Gram and B. Fonnesbech Vogel 2003. Genetic variability among isolates of *Listeria monocytogenes* from food products, clinical samples and processing environments, estimated by RAPD-typing. *Int. J. Food Microbiol.* **84**, 285-297

Mejlholm, O., N. Bøknæs and P. Dalgaard 2005. Shelf-life and safety aspects of chilled cooked and peeled shrimps (*Pandalus borealis*) in modified atmosphere packaging *J. Appl. Microbiol.* **99**, 66-76.

Nilsson, L., H.H. Huss and L. Gram 1997. Inhibition of *Listeria monocytogenes* on cold-smoked salmon by nisin and carbon dioxide atmosphere. *Int. J. Food Microbiol.* **38** 217-228.

Nilsson, L., L. Gram and H.H. Huss. 1999. Growth control of *Listeria monocytogenes* on cold-smoked salmon using a competetive lactic acid bacterial flora. *J. Food Prot.* **62** 336-342

Nilsson, L., T. Beck Hansen, P. Garrido, C. Buchrieser, P. Glaser, S. Knøchel, L. Gram and A. Gravesen 2005. Growth inhibition of *Listeria monocytogenes by a* nonbacteriocinogenic *Carnobacterium piscicola. J. Appl. Microbiol.* **98**, 172-183.

Nilsson, L., Y. Chen, M.L. Chikindas, H.H. Huss, L. Gram and T.J. Montville 2000. Carbon dioxide and nisin act synergistically on the cytoplasmic membrane of *Listeria monocytogenes. Appl. Environ. Microbiol.* **66** 769-774.

Nilsson, L., Y. Ng, J.N. Christiansen, D. Jacobsen, B.L. Jørgensen and L. Gram 2004. The importance of a plasmid-encoded class IIa bacteriocin for inhibition of *Listeria monocytogenes* by *Carnobacterium piscicola* strain A9b. *J. Appl. Microbiol.* **96**, 133-143.

Ross, T., P. Dalgaard and S. Tienungoon 2000. Predictive modelling of the growth and survival of *Listeria* in fishery products. *Int. J. Food Microbiol.* **62**, 231-245.

Vogel, B.F., B. Ojeniyi, P. Ahrens, L. Due Skov, H.H. Huss and L. Gram 2001b. Elucidation of *Listeria monocytogenes* contamination routes in cold-smoked salmon processing plants detected by DNA-based typing methods. *Appl. Environ. Microbiol.* **67**, 2586-2595

Vogel, B.F., L.V. Jørgensen, B. Ojeniyi, H.H. Huss and L. Gram 2001a. Diversity of *Listeria monocytogenes* isolates found in cold-smoked salmon from different smoke houses assessed by randomly amplified polymorphic DNA analyses. *Int. J. Food Microbiol.* **65**, 83-92.

Vogel, B.F, V. Fussing, B. Ojeniyi, L. Gram and P. Ahrens 2004. High-resolution genotyping of *Listeria monocytogenes* by fluorescent amplified-fragment length polymorphism analysis as compared to typing by PFGE, RAPD, ribotyping and PCR-RFLP. *J. Food Prot.* **67**, 1656-1665.

Wulff, G., L. Gram, P. Ahrens and B.F. Vogel 2005. Dection of dminating presistent *Listeria monocytogenes* sub-types frequently occurring in fish slaughter and smoke houses (in prep.)